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Protein Composition of the Cell Wall and Cytoplasmic Membrane of *Escherichia coli*

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Envelope preparations obtained by passing *Escherichia coli* cells through a French pressure cell were separated by sucrose density gradient centrifugation into two distinct particulate fractions. The fraction with the higher density was enriched in fragments derived from the cell wall, as indicated by the high content of lipopolysaccharide, the low content of cytochromes, and the similar morphology of the fragments and intact cell walls. The less-dense fraction was enriched in vesicles derived from the cytoplasmic membrane, as indicated by the enrichment of cytochromes, the enzymes lactic and succinic dehydrogenase and nitrate reductase, and the morphological similarity of the vesicles to intact cytoplasmic membrane. Both fractions were rich in phospholipid. The protein composition was compared by mixing the cytoplasmic membrane-enriched fraction from a ^{3}H-labeled culture with the cell wall-enriched fraction from a ^{14}C-labeled culture and examining the resulting mixture by gel electrophoresis. Thirty-four bands of radioactive protein were resolved; of these, 27 were increased two- to fourfold in the cytoplasmic membrane-enriched fraction, whereas 6 were similarly increased in the cell wall-enriched fraction. One of the proteins which is clearly localized in the cell wall is the protein with a molecular weight of 44,000, which is the major component of the envelope. This protein accounted for 70% of the total protein of the cell wall, and its occurrence in the envelope from spheroplasts suggests that it is a structural protein of the outer membranous component of the cell wall.

The cell wall of *Escherichia coli* is a complex, multilayered structure which cannot be resolved by simple means. The structure of the wall has been examined by electron microscopy of fixed, sectioned preparations (6, 16) and more recently by the technique of freeze-etching (1, 18); these studies, however, have provided little concrete evidence concerning the localization of the various types of macromolecular compounds which have been identified as components of the *E. coli* envelope. A combination of selective extraction techniques and electron microscopy were used by Weidel et al. (23) and Martin (12, 13) to show that the outermost layers of the envelope contained a complex mixture of lipid, protein, and polysaccharide. The isolated murein (peptidoglycan) component prepared by sodium dodecyl sulfate extraction contained bound protein (10). This protein has now been isolated and characterized by Braun et al. (2-4), who found this to be a protein of low molecular weight (approximately 7,000) which is covalently bound to diaminopimelic acid residues of the murein.

The innermost layer of the cell wall, as observed in sectioned preparations, is thought to be the locus of the murein (peptidoglycan) component of the wall, since this layer disappears when cells are converted to spheroplasts by the action of penicillin or lysozyme (6, 16). It is generally impossible to observe the lipopolysaccharide (LPS) component of the cell wall directly in sectioned preparations; however, a combination of serological studies with electron microscopy indicates that much, if not all, of this compound is localized in the outermost layers of the cell wall (15, 21).

The present report is concerned with the separation of crude envelope preparations into two fractions, one of which is enriched in cell wall fragments, whereas the other is enriched in fragments derived from the cytoplasmic membrane. A comparison of the chemical composition and morphology of these fractions provides some

evidence about the structure and composition of the *E. coli* cell wall.

MATERIALS AND METHODS

Cultures and growth conditions. The bacterial strain used in this study was *E. coli* J-5, a gift of Edward Heath. This strain is a mutant derived from *E. coli* 0111_{B4} which is lacking the enzyme uridine diphosphate-galactose-4-epimerase. This strain is unable to form complete LPS in the absence of added galactose (7). When galactose is added to the culture medium, normal LPS is formed, and labeled galactose is incorporated exclusively into LPS (7, 11). The minimal salts medium and the growth conditions were the same as previously described (20) with the exception that 1% Casamino Acids neutralized with NaOH was used as carbon source, as indicated below.

Incorporation of labeled galactose into LPS. The most reproducible incorporation of labeled galactose into the cell wall fraction was obtained when cells were induced by growth in the presence of unlabeled galactose before addition of the labeled galactose and when the labeling period was followed by a "chase" of unlabeled galactose. Overnight cultures were grown on minimal salts containing the appropriate carbon source plus 1 mM galactose. Overnight cultures (50 ml) were then centrifuged at room temperature, and the pellet was suspended in 500 ml of fresh medium containing the same carbon source plus 30 μCi of ^{14}C-galactose (specific activity, 8.2 mCi/mmole) and grown for 1.5 hr on Casamino Acids or glucose or 2.5 hr on succinate. The medium was then made 5 mM by the addition of concentrated unlabeled galactose, and growth was allowed to continue for 1 additional hr. In some cases, the protein was also labeled with ^{3}H-leucine and ^{3}H-tyrosine as previously described (20). In cases in which nitrate reductase was to be measured, 1 mM KNO_3 was also added to both the starter culture and the final culture.

Breakage of cells and isolation of envelope fractions. Cells were harvested, treated in a blendor to remove extraneous material, and broken by passage twice through a French pressure cell exactly as previously described (20). After addition of $MgCl_2$ and removal of debris by low-speed centrifugation, the envelope fraction was obtained by centrifugation in a Spinco 50Ti rotor for 45 min at 50,000 rev/min. The pellet from this centrifugation, which is designated as the crude envelope fraction, was suspended to a final protein concentration of 10 to 30 mg/ml in 0.01 M *N*-2-hydroxyethylpiperazine-*N*'-2'-ethane sulfonic acid (HEPES) buffer (*p*H 7.4). The treatment of the crude envelope fraction with Lubrol WX (20) was omitted, since this causes inactivation of some membrane-bound enzymes. Portions (1 ml) of the crude envelope suspension were then layered on 28-ml sucrose gradients prepared in the same buffer. The exact sucrose gradients are given below. The gradients were centrifuged for 16 hr in a Spinco SW 25.1 rotor at 25,000 rev/min. The gradients were fractionated by pumping the solution out through a metal capillary tube inserted into the bottom of the gradients. In the case of the discontinuous gradients, the fractions containing material absorbing at 280 nm were pooled and diluted twofold with a solution containing 0.1 mM $MgCl_2$, 0.25 M sucrose, 0.01 M NaCl, and 0.02 M phosphate buffer (*p*H 6.8). The particulate material was recovered by centrifugation in the Spinco 50Ti rotor for 1.5 hr at 50,000 rev/min. The samples were suspended in a small volume of distilled water.

Preparation of samples for electron microscopy. Samples of intact cells were centrifuged for 5 min in a Beckman Microfuge. The amount of sample was adjusted to give pellets less than 0.1 mm thick. The supernatant fluid was decanted, and 6% glutaraldehyde in 0.05 M phosphate buffer (*p*H 7.0) containing approximately 0.1 mM $CaCl_2$ was added to the Microfuge tube. The solution was allowed to stand for 2 hr at room temperature, the samples were centrifuged, and the supernatant fluid was decanted. The pellets were then washed at 0 C with at least four changes of the same buffer without glutaraldehyde over a period of at least 8 hr, with recentrifugation as necessary to prevent resuspending. The samples were then fixed overnight by adding 1% OsO_4 in the same buffer to the Microfuge tubes. The buffer with OsO_4 was decanted, and the samples were dehydrated through 90% ethanol in the Microfuge tubes. The tips of the tubes were cut off just below the pellets, and the pellets were loosened from the tubes by prodding with a dull pencil point. The dehydration was continued, and the pellets were embedded in Epon 812, sectioned, and stained with lead citrate and uranyl acetate as previously described (19). Spheroplasts were fixed in the same fashion, except that 20% sucrose (w/v) was added to the fixation and washing buffers as an osmotic stabilizer. Spheroplast membrane preparations, crude envelope preparations, and the cell wall-enriched fraction were fixed in the same fashion, except that the speed of the Microfuge was increased by operation at 150 v from a variable transformer and that the time of centrifugation was increased to 10 min. The membrane-enriched fraction was fixed by mixing the sample with an equal volume of 12% glutaraldehyde in the phosphate buffer described above and by centrifuging immediately in the Microfuge for 10 min at 150 v. The supernatant fluid was decanted and replaced with fresh 6% glutaraldehyde in the same buffer. Since glutaraldehyde effectively stabilizes these pellets, the pellets were removed from the Microfuge tubes after 2 hr in glutaraldehyde instead of at the dehydration stage. The same fixation and washing schedule was followed.

Preparation of spheroplast envelopes. A membrane fraction from penicillin spheroplasts was isolated by a modification of the procedure of Kaback and Stadtman (9). A log-phase culture (600 ml) grown on minimal salts medium plus 0.5% glucose as the carbon source (20) was added to 1 liter of the same medium containing 20% (w/v) sucrose. Concentrated $MgCl_2$ was added to bring the final concentration of Mg^{2+} to 1 mM, and penicillin G was added to give a final concentration of 10^3 units/ml. This culture was incubated until spheroplast formation was complete, as judged by phase microscopy, which required about 3 hr. The culture was harvested by centrifugation for

10 min at 10,000 rev/min in a Sorvall GSA rotor, and the spheroplasts were washed once by suspension in 0.05 M phosphate buffer (*p*H 7.0) containing 20% sucrose. The mixture was again centrifuged, and the spheroplasts were suspended in 150 ml of a solution containing 20% sucrose, 60 μg of crystalline lysozyme per ml, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM KCl, and 20 mM tris(hydroxymethyl)-aminomethane (Tris) buffer (*p*H 7.8). This suspension was incubated for 10 min at room temperature with stirring. The suspension was then centrifuged for 10 min in a Sorvall SS-34 rotor at 15,000 rev/min, and the pellet was suspended in 150 ml of Tris buffer (*see above*) containing 10 mM EDTA and 1 mg each of pancreatic ribonuclease and deoxyribonuclease. A 1.5-ml amount of 1 M $MgCl_2$ was then stirred into this viscous suspension, and stirring was continued until the viscosity decreased. The membrane fraction was recovered by centrifugation at 20,000 rev/min in a Sorvall SS-34 rotor. The pellet was washed three times by suspension in Tris buffer containing 10 mM EDTA. The final preparation was freed of whole cells by centrifuging onto a cushion of 2 M sucrose, and the interface band was diluted and centrifuged as above.

Chemical and radioactive assays. Total protein, radioactive protein, phospholipid, succinic dehydrogenase, and lactic dehydrogenase were assayed as previously described (19, 20). Nitrate reductase was measured with the use of reduced benzyl viologen as an electron donor, by the method of Itagaki and Taniguchi (8) except that the reaction was stopped by the addition of three drops of 30% H_2O_2 rather than by shaking.

Reagents. Sucrose (special enzyme grade) for use in fixation and preparation of gradients and benzyl viologen were obtained from Mann Research Laboratories, Inc., New York, N.Y. Glutaraldehyde was obtained from Eastman Organic Chemicals, Rochester, N.Y., and was used without further purification. Other biochemicals were obtained from Calbiochem, Inc., Los Angeles, Calif.

Gel electrophoresis. The preparation of lipid-free membrane protein and its examination by gel electrophoresis were carried out as previously described (20).

RESULTS

Rationale behind the fractionation procedure. The usefulness of sucrose gradients for the isolation of fractions enriched in the cell wall and the cytoplasmic membrane was suggested by electron microscopic examination of envelope preparations obtained by breaking cells in a French pressure cell. When cells were broken in the presence of high levels of salts, as for example in the minimal salts medium, the resulting fragments were large and often composed of both the cell wall and the cytoplasmic membrane. However, when this procedure was repeated in media of low ionic strength containing a small amount of EDTA (20), the fragments were much smaller. Such a preparation is shown in Fig. 1. This preparation consists primarily of two types of envelope fragments: very small closed vesicles composed of a single unit membrane and larger fragments with the five-layered structure characteristic of the *E. coli* cell wall. Examples of these two types of structure are illustrated also in Fig. 5A and in the insert in Fig. 6. The fragments which resemble the cell wall are usually not present as closed vesicles, but as C-shaped open structures. These fragments are clearly derived from the cell wall, since they contain both the membranous component of the wall and the inner layer of murein (glycopeptide) described by DePetris (6) and by Murray et al. (17). The presence of this rigid structure explains the inability of these fragments to vesiculate. The smaller vesicles by the same reasoning must be derived primarily from the cytoplasmic membrane which lacks such a rigid layer. Since carbohydrate is a dense material with respect to lipoprotein, it was felt likely that the wall fragments should have a higher density than the membrane vesicles.

To obtain unequivocable identification of the cell wall and the cytoplasmic membrane in fractionation studies, it was also necessary to choose biochemical markers for the two fractions. LPS was chosen as a marker for the cell wall, since a number of other techniques had indicated that much, if not all, of this compound was located in the cell wall (15, 21). Succinic dehydrogenase was chosen as a likely marker for the cytoplasmic membrane.

Fractionation of envelope preparations on sucrose gradients. Figure 2 illustrates the distribution of radioactive protein, succinic dehydrogenase, and LPS after centrifugation of a crude envelope preparation on a continuous sucrose gradient. Two visible bands of turbidity were observed in the tubes at the locations indicated by the arrows. The upper band was faint but with a strong reddish cast, whereas the bottom band scattered light strongly but was almost white in color. The lower band contained most of the galactose label, indicating the presence of most of the LPS. Some succinic dehydrogenase was found in the lower band, but the major peak of succinic dehydrogenase activity coincided with the upper band. A considerable amount of soluble protein remained near the top of the gradient, and this also contained some succinic dehydrogenase. This was not surprising, since it was observed in preliminary experiments that succinic dehydrogenase is partially solubilized from the membranes in media of low ionic strength. Since these preparations were not

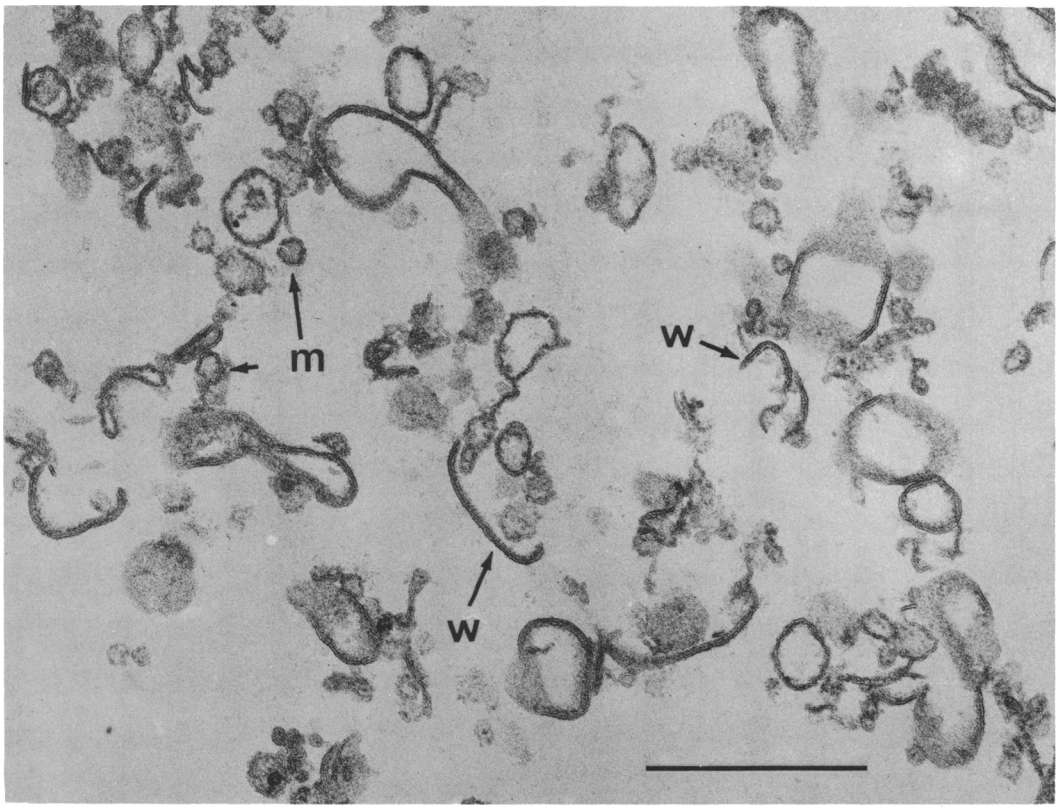

FIG. 1. *Unfractionated crude envelope preparation. To simulate the appearance of the envelope fractionation, the crude preparation was washed once by suspension in 0.25 M sucrose in 0.01 M HEPES buffer (pH 7.4) and centrifugation. Symbols: m, small membrane vesicles; W, cell wall fragments.* ×60,000. *Bar, 0.5 μm.*

treated with lubrol or washed extensively, it is probable that some of this soluble protein is cytoplasmic protein.

To obtain more material for analysis, a discontinuous gradient (Fig. 3) was employed. The sucrose concentrations are indicated in Fig. 3. The two bands from this gradient were collected and examined for morphology and biochemical properties. The biochemical results are summarized in Tables 1 and 2. The lower band contained most of the labeled LPS, and the enrichment of this component indicated that the fraction was derived primarily from the cell wall. This lower band will be subsequently designated the "wall-enriched fraction." The upper band showed a similar enrichment in the enzymes succinic dehydrogenase, lactic dehydrogenase, and nitrate reductase, indicating that it was derived from the cytoplasmic membrane. This upper band will subsequently be designated the "cytoplasmic membrane-enriched fraction." The recovery of succinic dehydrogenase is high, suggesting some activation of this enzyme in the gradients. However, the recoveries of other enzymes, the galactose label, and the phospholipid suggest that the recovery of each organelle is approximately 60%. Both fractions contained phospholipid, the phospholipid content of the cell wall indicating that the membranous component of the wall is in fact a lipoprotein membrane similar in overall composition to the cytoplasmic membrane and to other biological membranes.

Figure 4 illustrates the relative distribution of cytochromes in the two fractions. The cytoplasmic membrane-enriched fraction appears to be enriched in both cytochrome *b* and cytochrome *a*. Exact quantitation could not be made, since the cell wall-enriched fraction scatters light much more strongly. The difference in light scattering probably reflects the difference in size of the two types of particles (Fig. 5 and 6).

The morphology of the fragments in these two fractions is illustrated in Fig. 5 and 6. Figure 5C illustrates a low-magnification micrograph of the cell wall-enriched fraction. This

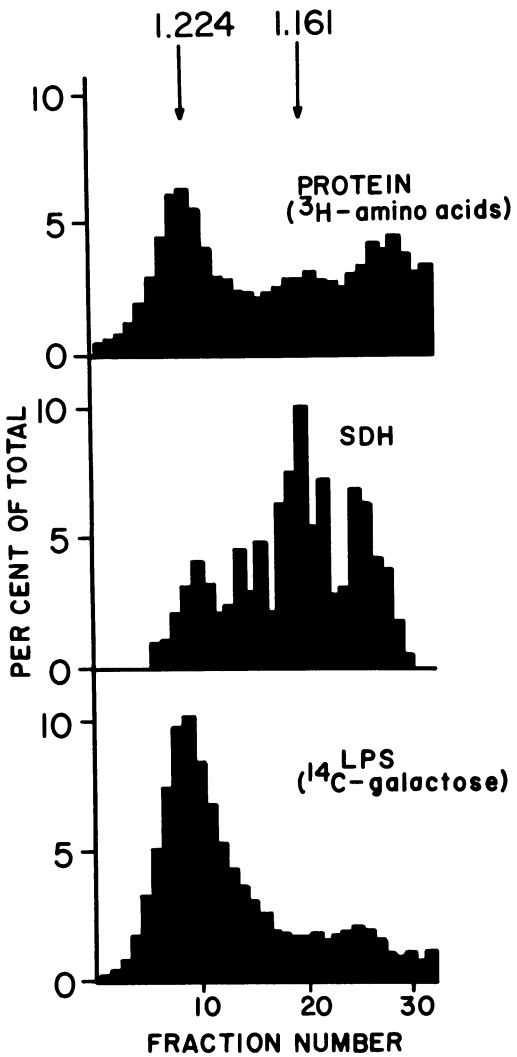

FIG. 2. *Fractionation of the crude envelope preparation on a continuous sucrose gradient. The gradient was linear from 20 to 55% (w/w) sucrose in 0.01 M HEPES buffer (pH 7.4). The cells were grown on succinate as a carbon source and labeled with ^{14}C-galactose, ^{3}H-leucine, and ^{3}H-tyrosine. The arrows indicate the location and density (25/40) of the two visible bands of turbidity.*

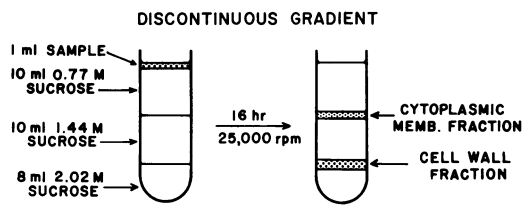

FIG. 3. *Discontinuous gradient used to obtain the cytoplasmic membrane-enriched and cell wall-enriched fractions. The sucrose was dissolved in 0.01 M HEPES buffer (pH 7.4).*

TABLE 1. *Fractionation of crude envelope preparation[a] on discontinuous sucrose gradient: specific activity or content[b]*

Fraction	Lipopolysaccharide[c]	Phospholipid	Succinic dehydrogenase	Lactic dehydrogenase	Nitrate reductase
Crude envelope preparation	0.99	0.34	0.60	0.53	0.40
Membrane-enriched fraction	0.51	0.63	5.24	1.75	1.68
Wall-enriched fraction	1.66	0.44	0.68	0.24	0.34

[a] Entire envelope fraction from two 500-ml late log-phase cultures grown on minimal salts plus 1% Casamino Acids as a carbon source. Nitrate reductase was induced by the addition of 1 mM KNO_3 after autoclaving.

[b] Culture was labeled with ^{14}C-galactose, and the LPS is expressed as counts per minute $\times 10^5$/mg protein. Phospholipid is expressed as μmoles lipid PO_4/mg protein. Enzymes are expressed as μmoles substrate converted/min/mg protein at room temperature.

[c] ^{14}C-galactose-labeled.

fraction consists almost entirely of large, open fragments with the characteristic morphology of the cell wall. This is shown in more detail in Fig. 5A and 5B, in which one such fragment is matched to the corresponding structure in an intact cell. On the other hand, the cytoplasmic membrane-enriched fraction consists primarily of very small vesicles (Fig. 6) which at higher magnification consist only of a single unit membrane (Fig. 6, insert). Many of the vesicles have a somewhat fuzzy appearance which is due, in part, to the osmotic shock as a result of isolation from the gradients and, in part, to the small size, since the diameter of many of the vesicles is about the same as the thickness of the Epon sections (30 to 50 nm).

Protein composition of the cell wall and cytoplasmic membrane. The wall-enriched and cytoplasmic membrane-enriched fractions described in Table 1 are obviously not pure. However, the distribution of LPS in the cell wall-enriched fraction indicates an enrichment of about threefold with respect to the cytoplasmic membrane, and the distribution of the various oxidative enzymes suggests that the cytoplasmic membrane-enriched fraction shows a similar enrichment with respect to the cell wall. A comparison of the protein profiles of these two fractions was made by gel electrophoresis; the results of this

TABLE 2. *Fractionation of crude envelope preparation on discontinuous sucrose gradient: total recovered*[a]

Fraction		Phospholipid[b]	Succinic dehydrogenase	Lactic dehydrogenase	Nitrate reductase	Protein
	counts/min					*mg*
Crude envelope preparation	4.28×10^6	14.7	25.9	22.9	17.3	43.2
Membrane-enriched fraction	0.24×10^6	3.0	24.6	8.2	7.9	4.7
Wall-enriched fraction	2.46×10^6	6.5	10.0	3.6	5.0	14.8
Per cent recovery (based on crude envelope)	63	65	134	52	74	45[c]

[a] Enzymes are expressed in total units recovered; 1 unit represents the conversion of 1 μmole of substrate/min at room temperature.

[b] Micromoles of phosphate.

[c] This low recovery is due to the presence of soluble or easily extractable protein which remains at the top of the gradient (see Fig. 2). If this is corrected for, the recovery is approximately 65%.

experiment are shown in Fig. 7. The experiment was done in the following way. Two identical cultures were grown in the minimal salts medium plus glucose. One culture contained ^{3}H-labeled leucine and tyrosine, whereas the other contained ^{14}C-labeled leucine and tyrosine. To insure uniform labeling, the labeled amino acids were added to both the overnight inoculum and to the final cultures. The two cultures were harvested, the cells were broken with the French pressure cell, and the crude envelope preparations were fractionated on discontinuous gradients (Fig. 3). The wall-enriched fraction from the ^{14}C-labeled culture was then mixed with the cytoplasmic membrane-enriched fraction from the ^{3}H-labeled culture, and this mixture was processed for gel electrophoresis as previously described (20). The counts in each gel slice are normalized by conversion to per cent of total counts, enabling the amount of protein labeled with each isotope to be compared directly. Of 34 proteins resolved on the gel, 27 were enriched two- to fourfold in the cytoplasmic membrane-enriched fraction, indicating that these proteins are components primarily of the cytoplasmic membrane. These proteins are indicated in Fig. 7 by the uncircled numbers above the peaks. One protein peak, identified as no. 21, was found in the same amount in both fractions. This represents either a protein which is shared in both the cell wall and the cytoplasmic membrane or two proteins of similar mobility, one in each fraction. The circled numbers (Fig. 7) indicate six proteins which were enriched two- to fourfold in the cell wall-enriched fraction. One protein which is clearly a component of the cell wall is protein no. 16, which is the major protein of the envelope fraction with a molecular weight of 44,000 (20). This protein accounts for 50% of the cell wall-enriched fraction, but only 13% of the cytoplasmic membrane-enriched fraction. This protein appears as a single, symmetrical protein in both fractions, supporting the previous indications (20) that this is a single protein species.

The clear distinction between proteins of the cytoplasmic membrane and cell wall permitted additional calculations of the amounts of the various proteins in the cell wall. Since many of the proteins shown in Fig. 7 are components of the cytoplasmic membrane, it was possible to subtract any ^{14}C counts in these peaks from the total ^{14}C counts in the cell wall-enriched fraction. This in effect corrects the cell wall-enriched fraction for any contaminating proteins

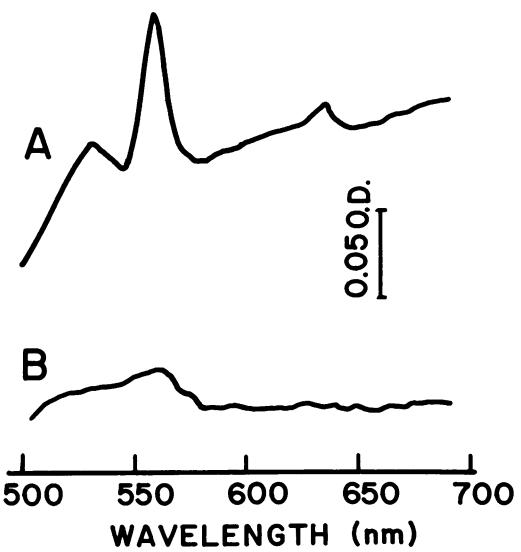

FIG. 4. *Difference spectra of cytoplasmic membrane-enriched fraction (A) and cell wall-enriched fraction (B). In each case, the final protein concentration was 0.62 mg/ml. Dithionite was added to the sample cuvet, and ferricyanide was added to the reference cuvet. Spectra are corrected for the baseline before this addition and were recorded in 1-cm cuvettes in a Cary 14 spectrophotometer.*

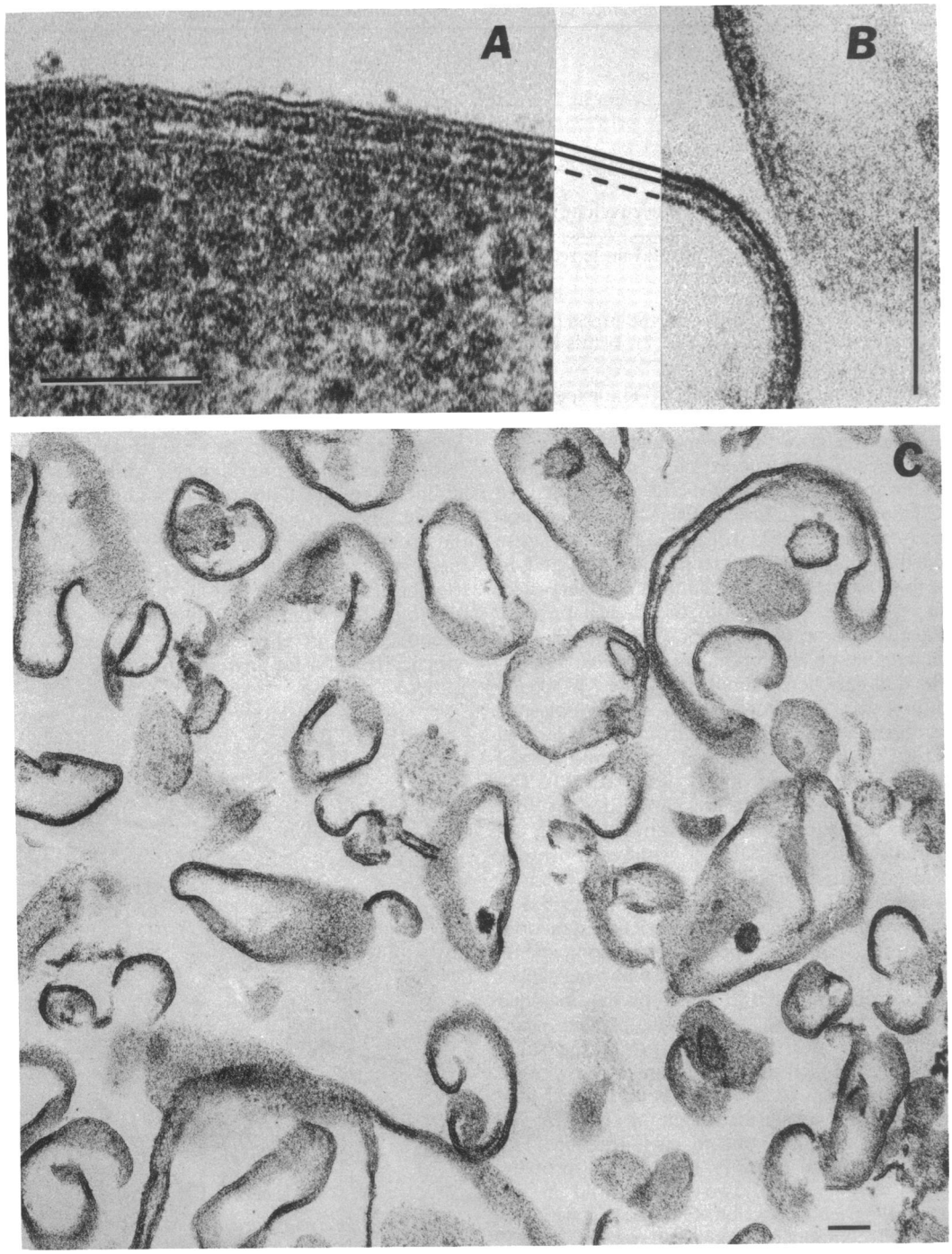

FIG. 5. *Electron microscopy of E. coli. A, Section through an intact cell of E. coli. ×240,000. B, Section through a typical fragment from the cell wall-enriched fraction. The inked lines indicate the corresponding structures in the intact and isolated cell wall. The dashed line indicates the murein layer. ×240,000. C, Appearance of the cell wall-enriched fraction at lower magnification. ×60,000. Bars, 0.1 μm.*

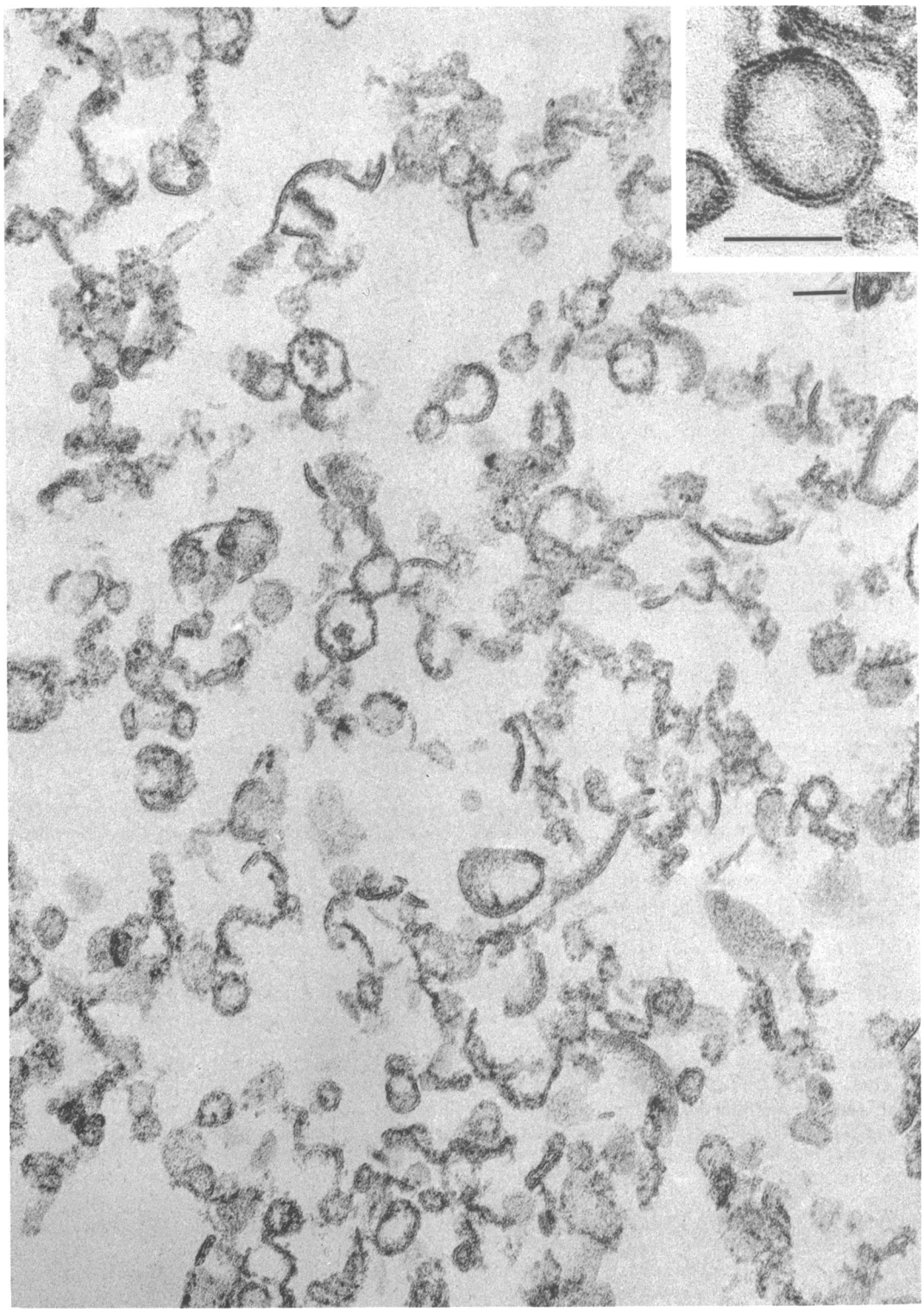

FIG. 6. *Appearance of the cytoplasmic membrane-enriched fraction. Inset illustrates one of the small vesicles at higher magnification.* ×*80,000. Inset* ×*180,000. Bars, 0.1 μm.*

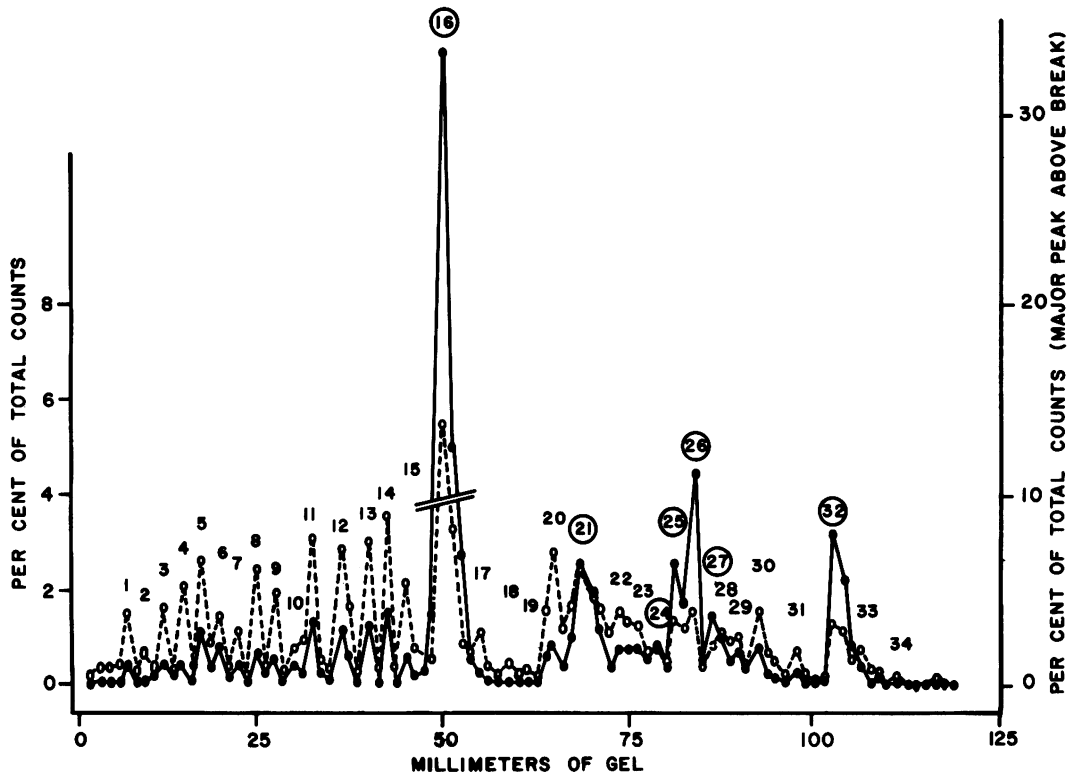

FIG. 7. *Protein profile of the cytoplasmic membrane-enriched fraction and cell wall-enriched fraction on 7.5% acrylamide gel. The ^{3}H-labeled cytoplasmic membrane-enriched fraction and the ^{14}C-labeled cell wall-enriched fraction were mixed before solubilization in DMF + HCl. The dashed line indicates ^{3}H counts and the solid line indicates ^{14}C counts. The numbers serve to identify the peaks, and the encircled numbers indicate proteins which appear to be components of the cell wall. The culture was grown with glucose as carbon source.*

derived from the cytoplasmic membrane. The amounts of the various cell wall proteins after this correction are shown in Table 3. After this correction, the protein in peak no. 16 accounts for 70% of the total protein of the cell wall.

Protein composition of spheroplast membranes. The data above indicate that the previously described protein of 44,000 molecular weight is the major protein of the cell wall. Therefore, it is of interest to attempt to localize this protein. Two loci of this protein are possible. First, this protein might be associated with the murein layer, since the studies of DePetris (6) indicated that this layer contained some form of "globules" of protein in addition to the peptidoglycan. Second, this protein could be a structural protein of the membranous component of the cell wall which would be analogous to the structural proteins of the membrane envelopes of animal viruses (22). Some light can be shed on this problem by the examination of the envelope preparations isolated from penicillin-induced spheroplasts, since the effect of penicillin is to prevent the formation of the murein layer (6, 17). Any protein normally associated with this layer should be lost on breakage of the spheroplasts or would possibly be repressed at the level of protein synthesis. The type of spheroplast preparation used in this study is illustrated in Fig. 8. In agreement with the results of Kaback and Stadtman (9), the preparation consisted primarily of large empty bags, many with a double membrane. Fig. 8A and 8B illustrate the similarity between these double membrane regions in the isolated envelopes and the corresponding structure in an intact spheroplast. This leaves little doubt that the membranous component of the cell wall is still present in the isolated envelope preparation, although it now cannot be distinguished from the cytoplasmic membrane. No structure resembling the murein layer of intact cell walls was observed in either the isolated envelope preparation or in the spheroplasts before lysis.

Figure 9 illustrates a scan of a stained gel of the spheroplast envelope protein preparation. The major protein component with a molecular weight of 44,000 is still present (arrow, Fig. 9).

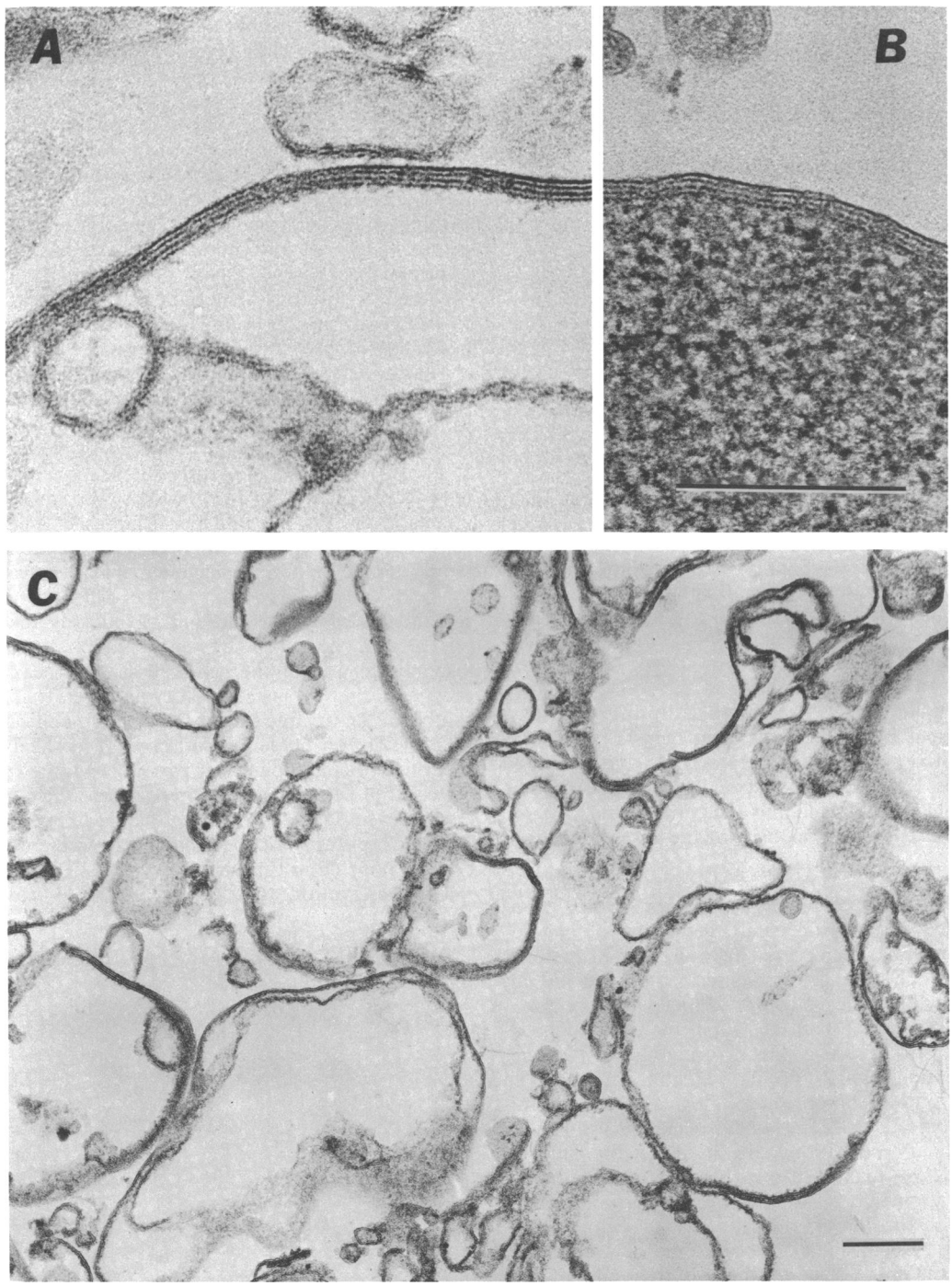

FIG. 8. *Electron microscopy of E. coli. A, Appearance of one of the double-membrane regions observed in the isolated spheroplast envelope preparation. B, A corresponding structure in an intact spheroplast. Note in both cases that the murein layer is completely absent. ×150,000. C, Appearance of the spheroplast envelope preparation at lower magnification. Note the frequent occurrence of double-membrane regions. ×45,000. Bars, 0.2 μm.*

TABLE 3. *Relative amounts of major cell wall proteins*

Peak no. (from Fig. 7)	Calculated per cent of total cell wall protein[a]
	%
16	70.4
21[b]	4.8
25	5.0
26	7.8
32	7.6

[a] ^{14}C counts which coincided with peaks which are clearly membrane proteins represent membrane contamination of the wall-enriched fraction. Results above were calculated from the gel shown in Fig. 8 by subtracting ^{14}C counts in peaks 1 through 15, 18 to 20, 22, 23, 28 to 31, 33, 34, and one half of peak 21 from the total. The amounts in the remaining peaks were calculated as a percentage of this new total ^{14}C. Approximations were made where peaks overlapped.

[b] Peak 21 appears to be either a common protein to both the wall and the membrane or an overlapping of identical peaks in the wall and membrane. Therefore, only one-half of the counts in this peak were assumed to be wall protein.

Comparison of amounts on the basis of isotope incorporation has not been made; however, on the basis of the intensity of the stained band in such gel scans, it appears that this protein is present in the same or even increased amounts relative to the envelope fraction from untreated cells (20). This fact strongly suggests that the protein is, in fact, the structural protein of the membranous component of the *E. coli* cell wall.

DISCUSSION

A procedure for separating the "outer membrane" and cytoplasmic membrane of spheroplasts has been reported by Miura and Mizushima (14). The preparation described in the present study has two distinct advantages over preparations with spheroplasts. First, the morphological features of the cell wall are preserved during isolation, which permits identification of the cell wall fraction by morphological and biochemical means. Second, intact cells grown under ordinary conditions are used as the starting material, making it possible to study the incorporation into the cell wall of components which are lost or altered during spheroplast formation. This preparational procedure may be important in studies of the biosynthesis of the murein layer.

The results shown in Tables 1 and 2 and Fig. 2 permit some tentative conclusions about the structure of the *E. coli* envelope. The innermost layer is the cytoplasmic membrane, which contains about one-third of the protein of the envelope and about one-half of the phospholipid; it is the locus of the respiratory enzymes of the cell. The cytoplasmic membrane is separated from the cell wall by a clear layer which is expanded to form the periplasmic space in plasmolyzed cells (5). The clear layer assumes a fixed minimum dimension in normal cells or in regions in which the cell wall and cytoplasmic membrane appear fused (for example, in Fig. 8B), suggesting that the layer is not empty space but is filled with carbohydrate or some other material which is not visible in conventional preparations for electron microscopy. Bayer and Remsen (1) observed a "fibrous material" which appears to be present in the clear layer, as observed in freeze-etched preparations.

The innermost layer of the cell wall contains the murein component of the wall, as illustrated by its loss during spheroplast preparation (16). It is likely that the protein described by Weidel et al. (23) and more recently by Braun et al. (2–4) is responsible for the "beaded" appearance of this layer (Fig. 5A and B). Braun and Rehn (2) demonstrated that this protein contains tightly bound lipid and suggested that it serves to bind the murine layer to the other outer constituents of the cell wall. It is unlikely that the protein described by these authors corresponds to any of the cell wall protein peaks shown in Fig. 7, since the murine layer is insoluble in the organic solvent used for solubilization of the envelope preparation. It is likely that this protein accounts for part of the approximately 15% of the envelope preparation which is insoluble in DMF + HCl (20). Experiments designed to test this possibility are presently being conducted in this laboratory.

The observation that the isolated cell wall

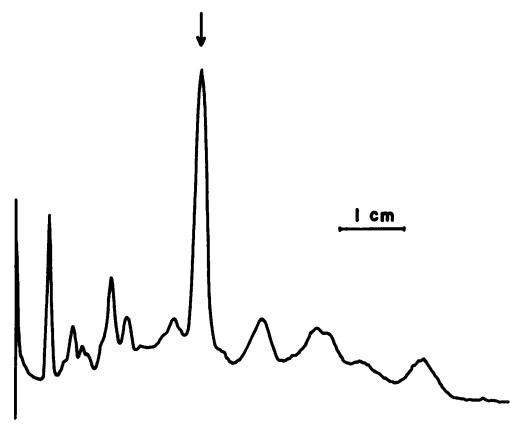

FIG. 9. *Optical density scan (620 nm) of a fixed, stained acrylamide gel of the isolated spheroplast envelope preparation. The arrow indicates the major protein with a molecular weight of 44,000. For a comparison with envelopes of intact cells, see reference 20.*

contains substantial amounts of both phospholipid and protein leaves little doubt that the outermost layers of the cell wall observed by conventional electron microscopy are in fact a lipoprotein membrane similar in basic respects to other biological membranes. The results obtained with spheroplast membranes suggest that this outer membrane may have a simple structure, being composed primarily of a single protein species; these results do not rule out the presence of small amounts of other proteins which confer to the cell surface the specificity necessary for sexual mating and for the action of phages and colicins. However, it would be an advantage to the cell for this outer membrane to be as simple as possible, since it must be assembled outside of the main permeability barrier of the cell. The results shown in Table 1 and Fig. 2 support the conclusion that the cell wall is the primary site of the lipopolysaccharide "O" antigen. It is likely that the lipid portion of the LPS is inserted into the phospholipid layer of this membrane, since interactions between isolated phospholipid and LPS are known to occur (24). Indeed, one can speculate that it is the presence of large amounts of polysaccharide inserted into the membrane of the cell wall which permits this membrane to be permeable to small solute molecules and eliminates the necessity for having specific permeation mechanisms on both the cell wall and the cytoplasmic membrane. It is likely that the structure of this membrane-polysaccharide complex is stabilized by divalent cations, which would explain the role of EDTA in promoting damage to the outer layers of the cell wall; the structure of the complex could also explain the release of LPS observed after EDTA treatment (11).

All gram-negative cell walls appear to contain some form of membranous component (16). It will be of interest to see whether envelopes isolated from other gram-negative bacteria also possess a single major protein species similar to the one reported in this study.

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